TROUBLESHOOTING GUIDE – WESTERN BLOT

Problem	Possible Cause	Possible Solutions
Weak or No Signal	Antibody concentrations too low	• Increase amount of antibody
	Primary antibody does not recognize antigen	• Check data sheets/references to verify that antibody does detect the antigen in the particular species
	Antibody may not recognize a denatured form of the antigen	• Dot blot native and denatured forms, then prove with antibody
	Secondary antibody may be inhibited	• HRP should not be used in conjunction with sodium azide or hemoglobin. Thimerosal can be substituted. • Biotinylated antibodies should not be used with milk or casein
	Inappropriate secondary antibody	• Check research/data sheets to make sure the secondary is directed against the host species and isotype of the primary antibody
	Constant freezing/thawing can affect antibody	• The antibody has most likely degraded. In the future, it is recommended that aliquots of smaller amounts be made.
	Antibody not stored as recommended	• The antibody has most likely degraded. Unfortunately a new vial must be used.
	Protein is undetectable	• Load more protein onto the gel • May be necessary to induce cells before harvest
	Detergents can disrupt protein binding	• Use a milder detergent such as Tween-20
	Too many proteins in sample cause competition	• Centrifuge lysate to enrich the sample with the protein of interest
	Insufficient Development Time	• Increase development time
	Sensitivity issues of detection system	• Use a more sensitive system- try ECL rather than colorimetric
	Wrong type of membrane	• Use PVDF membranes for a maximum signal. Remember to use more stringent blockers.

Problem	Possible Cause	Possible Solutions
Diffuse Background Staining	Insufficient Blocking	• Block overnight • Increase amount of blocker • Try a different blocker • Repeat with a freshly made blocker • Are the antibodies in the buffer too?
	Inadequate Washing	• Increase stringency and number of washes
	Contaminated solutions, membrane mishandling, and/or dirty gel plates can lead to spots and streaks	• Be aware of potential contaminants- use clean equipment, fresh solutions, wear gloves
Non-Specific Bands	Incomplete protein reduction causing non-specific bands with a high molecular weight	• Use a stronger and more stable reducing agent such as DTT over _-mercaptoethanol • Purified protein is more prone to aggregation. A high molecular weight laddering pattern may be showing
	Sample degradation causing non-specific bands with low molecular weights	• Add a sample buffer with protease inhibitor immediately after harvest • Always use fresh samples • Store samples at -70°C or below
	Protein overloading	• Use less protein
	Loading whole cell homogenate	• Use cell lysate supernatant for non-membrane bound proteins
	Antibody concentration too high	• Decrease concentration
	Over-incubation with secondary antibody enzyme substrate	• Decrease staining time
	Antibody-antigen cross reactions	• Determine which bands are being caused by the secondary antibody by probing the membrane
	Additional proteins are being recognized by polyclonal antiserum and causing bands	• Use a monoclonal or affinity purified antibody
	Bands generated by recognition of homologous amino acid sequences	• Use a monoclonal antibody specific for a different epitope
	Extended incubation periods	• One hour should be sufficient for most proteins • Try raising the temperature, rather than increasing the time

Problem	Possible Cause	Possible Solutions
Poor Protein Transfer	Protein won't transfer	• Use a nylon membrane • Increase field strength • Increase transfer time
	Protein transfers through membrane	• Shorten transfer time • Use a double layer of membrane • Use a PVDF membrane
	Protein lost from membrane after transfer	• Treat with isopropanol for one minute • Allow membrane to completely dry before blocking
	Protein binding is blocked	• Use brand new nitrocellulose sheets • Always wear gloves when handling membranes